Immunity, Vol. 10, 117–125, January, 1999, Copyright ©1999 by Cell Press

BLNK Required for Coupling Syk to PLCγ2 and Rac1-JNK in B Cells

Masamichi Ishiai,* Mari Kurosaki,* Rajita Pappu,† Katsuya Okawa,‡ Irina Ronko,† Chong Fu,† Masao Shibata,§ Akihiro Iwamatsu,‡ Andrew C. Chan,† and Tomohiro Kurosaki*‖

*Department of Molecular Genetics
Institute for Liver Research
Kansai Medical University
Moriguchi 570-8506
Japan

†Center for Immunology
Division of Rheumatology
Departments of Internal Medicine and Pathology
Howard Hughes Medical Institute
Washington University School of Medicine
St. Louis, Missouri 63110

‡Central Laboratories for Key Technology
Kirin Brewery Company
Yokohama 236-0004
Japan

§Medical Biological Laboratories Ina Laboratory
Nagano 396-0002
Japan

Summary

Signaling through the B cell receptor (BCR) is essential for B cell function and development. Despite the key role of Syk in BCR signaling, little is known about the mechanism by which Syk transmits downstream effectors. BLNK (B cell LiNKer protein), a substrate for Syk, is now shown to be essential in activating phospholipase C (PLC)γ2 and JNK. The BCR-induced PLCγ2 activation, but not the JNK activation, was restored by introduction of PLCγ2 membrane-associated form into BLNK-deficient B cells. As JNK activation requires both Rac1 and PLCγ2, our results suggest that BLNK regulates the Rac1-JNK pathway, in addition to modulating PLCγ2 localization.

Introduction

B cell receptor (BCR) engagement triggers complex cascades of biochemical events that culminate in gene transcription, cellular proliferation, and differentiation. The BCR utilizes sequential activation of at least three types of cytoplasmic protein tyrosine kinases (PTKs), Src-PTK, Syk, and Btk to regulate downstream effectors. Deficiencies of these three families of PTKs result in defective or aberrant B cell function and development (Pleiman et al., 1994; DeFranco, 1997; Kurosaki, 1997; Reth and Wienands, 1997). Thus, characterization of the substrates of these activated PTKs is a prerequisite for understanding the details of BCR-mediated signal transduction.

Two direct consequences of protein tyrosine phosphorylation have been demonstrated in the BCR signaling system. First, tyrosine phosphorylation can activate enzymes. Examples are Syk, which is phosphorylated for full kinase activity (Hutchcroft et al., 1991; Yamada et al., 1993), and phospholipase C (PLC)γ2, which upon activation generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Nishibe et al., 1990; Hempel and DeFranco, 1991; Coggeshall et al., 1992). Second, tyrosine phosphorylation creates sites for binding to proteins with SH2 domains. One example is the interaction between Igα/Igβ and Syk upon BCR stimulation. Syk SH2 domains bind to the doubly phosphorylated tyrosine residues of Igα/Igβ (Kurosaki et al., 1995; Rowley et al., 1995). In addition, tyrosine phosphorylation permits multimeric protein complex formation. For instance, Grb2 adaptor protein consists of an SH2 domain surrounded by two SH3 domains (Lowenstein et al., 1992). Grb2 SH2 domain binds to specific phosphotyrosyl residues on its target protein and the SH3 domain binds to proline-rich motifs on separate sets of target proteins such as Sos and Cbl (Saxton et al., 1994; Smit et al., 1994; Fukazawa et al., 1995; Panchamoorthy et al., 1996; Harmer and DeFranco, 1997). Thus, upon BCR stimulation, the SH2 domain of Grb2 is thought to interact with tyrosine-phosphorylated membrane proteins, thereby bringing Sos to the plasma membrane.

While much has been learned as to the relationship between the BCR-associated PTKs and downstream effectors, the molecular mechanism by which these PTKs regulate downstream events remains unclear. Analogous to receptor tyrosine kinases (Pawson and Schlessinger, 1993), it has been thought that many signaling molecules directly bind phosphorylated tyrosine residues on the cytoplasmic domains of Igα and Igβ and/or on the BCR-associated PTKs. However, this does not appear to be a feature of the coupling mechanism to downstream signaling pathways. Attention instead has focused on adaptor proteins, one of which is BLNK (alternatively named SLP-65) (Fu and Chan, 1997; Fu et al., 1998; Wienands et al., 1998). BLNK is a B cell–specific protein that is phosphorylated by Syk after BCR ligation. BLNK, like SLP-76 (Jackman et al., 1995), comprises a COOH-terminal SH2 domain, a central proline-rich region that binds to Grb2 SH3 domains, and multiple tyrosine phosphorylation sites.

We have investigated the mechanisms by which Syk mediates downstream effectors in B cells. Since BLNK interacts with a variety of downstream effector proteins including PLCγ2, Vav, Grb2, and Nck (Fu and Chan, 1997; Fu et al., 1998; Wienands et al., 1998), we hypothesized that BLNK functions as a master substrate to interface Syk with multiple downstream effectors. We now report the consequences of disruption of BLNK on the BCR-mediated responses and demonstrate a critical role for BLNK in activation of PLCγ2 and Rac1-JNK, but not Ras.

‖To whom correspondence should be addressed (e-mail: kurosaki@mxr.meshnet.or.jp).

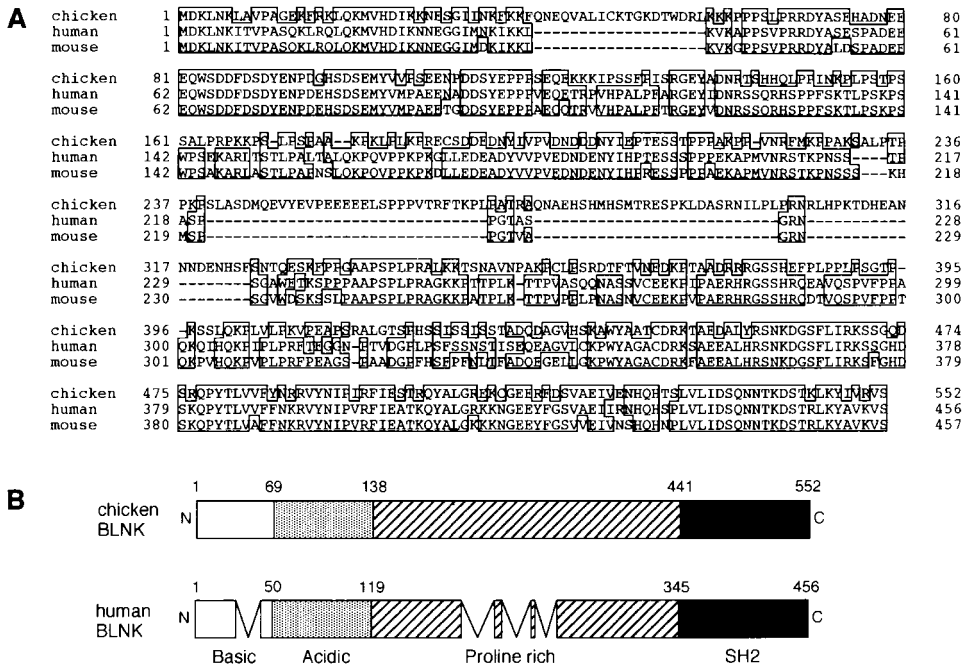

Figure 1. The Gene for Chicken pp80 Encodes BLNK

(A) Alignment of the BLNK protein. The sequences of chicken (longer form), human (longer form; pp70) (Fu et al., 1998), and mouse (Fu et al., 1998) were aligned by the Clustal W program (Thompson et al., 1994). Identical amino acids are indicated by the box. The shorter form of chicken BLNK lacks 20 amino acids (positions 38–57). Peptide sequences obtained from microsequences were as follows (amino acid number): LAVPAGEK (7–14), KPPPSLPRR (61–69), DNRTSHHQ (140–147), VPEAPSRAL (408–416), AWYAATCDRK (441–450), DGSFLIRK (462–469), CGEERFDSVAEIVEN (510–524). (B) Schematic diagrams of human and chicken BLNK. Structural domains of BLNK are shown.

Results

Chicken pp80 That Is Tyrosine Phosphorylated by BCR Ligation Is BLNK

Our previous studies have shown that BCR-evoked PLCγ2 activation is a downstream event of Syk in DT40 B cells (Takata et al., 1994). These genetic data, however, do not necessarily indicate that PLCγ2 is directly regulated by Syk in B cells. Indeed, while Syk can phosphorylate PLCγ1 in COS cells (Law et al., 1996), expression of a functional BCR, Fyn, and Syk in non-lymphoid cells does not induce PLCγ phosphorylation or increased intracellular Ca^{2+} ($[Ca^{2+}]i$) upon receptor stimulation (Richards et al., 1996). These reconstitution studies suggest the existence of B cell–specific protein(s) that may link Syk with PLCγ2 activation. Thus, we purified tyrosine-phosphorylated proteins from activated DT40 cells by affinity purification protocol based on their ability to bind an anti-phosphotyrosine MAb.

Among several purified proteins, four internal peptide sequences obtained from microsequencing of pp80 were very homologous to those of human and mouse BLNK (Figure 1A). A chicken cDNA was cloned by combination of RT-PCR method and library screening (see Experimental Procedures). Two PCR products that differed by the presence of the insertion of 20 amino acids at their N termini were detected (Figure 1 legend). Both forms contain all of the tyrosine phosphorylation sites, the proline-rich domain, and the C-terminal SH2 domain, similar to human and mouse BLNK (Figure 1B). Thus, we conclude that pp80 phosphoprotein is a chicken homolog of BLNK.

BLNK Is Required for BCR-Induced PLCγ2 Activation

To address the function of BLNK, we established DT40 B cells deficient in BLNK by gene-targeting method. Lack of BLNK expression was confirmed by Northern and Western analyses (Figures 2A and 2B). The level of cell surface expression of BCR on BLNK-deficient DT40 clone was essentially the same as that of parental DT40 cells (Figure 2C). Transcript of chicken SLP-76 in DT40 cells could not be detected by RT-PCR method (data not shown).

Comparison of the BCR-induced overall tyrosine phosphorylation between wild-type and BLNK-deficient DT40 cells did not exhibit significant changes, except that the band corresponding to BLNK itself was absent in the mutant cells (Figure 2D). These results suggest that the BCR-associated PTKs such as Lyn and Syk are activated normally in the absence of BLNK.

One of the hallmarks of the BCR-induced signaling is calcium mobilization. Fura 2-loaded wild-type and BLNK-deficient DT40 cells were stimulated with anti-BCR MAb, M4, and the rise of $[Ca^{2+}]i$ was measured. As shown in Figure 3A, no increase in $[Ca^{2+}]i$ was detected in BLNK-deficient cells. Consistent with the calcium defect, BLNK-deficient DT40 cells abolished the tyrosine phosphorylation of PLCγ2 (Figure 3C), resulting in the loss of IP_3 generation (Figure 3B) upon receptor stimulation. These defects were restored by reexpression of chicken BLNK (longer form) in the BLNK-deficient cells (Figures 2B, 3A, 3B, and 3C). These data indicate that BLNK is required for coupling Syk to PLCγ2 activation in DT40 B cells.

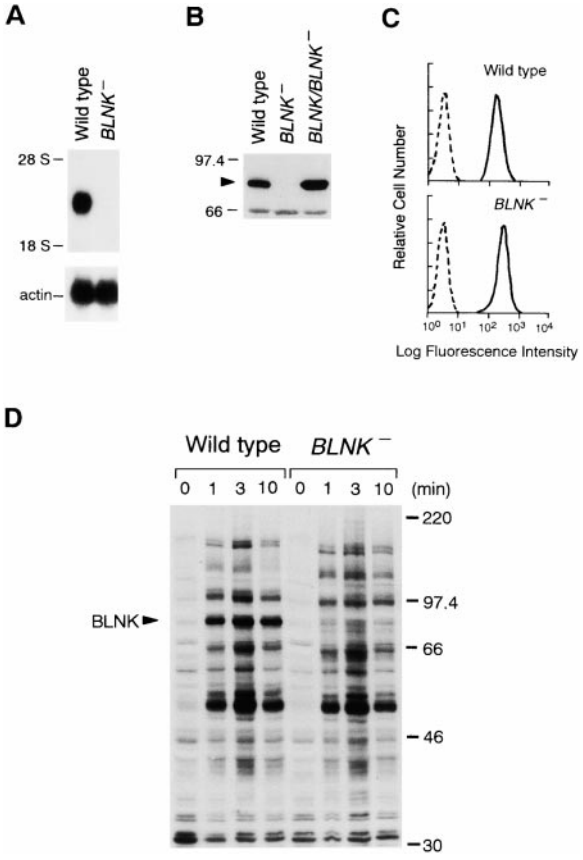

Figure 2. Disruption of the BLNK Gene in Chicken DT40 B Cells

(A) Northern blot analysis of BLNK RNA expression. RNAs prepared from wild-type and BLNK-deficient DT40 cells were separated in 1.2% formaldehyde gel, blotted, and probed with ^{32}P-labeled cDNAs chicken BLNK (top) or β-actin (bottom). The positions of the 28S and 18S rRNA are shown.

(B) Protein expression analysis of BLNK. Total cell lysate (2.5×10^6 cells) were prepared and analyzed by Western blotting using anti-BLNK Ab. Transformant of chicken BLNK cDNA (longer form) into BLNK-deficient DT40 cells is indicated as BLNK/*BLNK*$^-$.

(C) Cell surface expression of BCR on wild-type and mutant cells. Unstained cells were used as negative controls (dot lines).

(D) Tyrosine phosphorylation in wild-type and BLNK-deficient DT40 cells. At the indicated time points after stimulation with M4 (4 μg/ml), whole-cell lysates prepared from 2.5×10^6 cells were loaded onto SDS-PAGE (8% gel) and analyzed by Western blotting with anti-phosphotyrosine Ab (4G10).

To formally demonstrate that BLNK acts upstream to PLCγ2 activation in the context of BCR signaling, we examined the effect of disruption of PLCγ2 on BLNK phosphorylation. Immunoprecipitation of BLNK from wild-type and PLCγ2-deficient DT40 cells demonstrated comparable levels of BLNK phosphorylation following BCR ligation (Figure 3D). Hence, tyrosine phosphorylation of BLNK by Syk likely lies upstream to that of PLCγ2.

Expression of PLCγ2 As a Membrane Chimera in BLNK-Deficient Cells Restored Calcium Mobilization

It has been previously shown that BLNK is translocated to the membrane fraction upon BCR ligation (Fu et al., 1998). Because PLCγ2 associates with BLNK in a receptor ligation-dependent fashion, we examined the translocation of PLCγ2 to the membrane fraction upon BCR aggregation in wild-type and BLNK-deficient B cells. While the stoichiometry of membrane-associated PLCγ2 was substantially enhanced following BCR ligation in wild-type DT40 cells, the translocation of PLCγ2 to the membrane fraction was markedly attenuated in BLNK-deficient cells (Figure 3E).

The above observations suggest that PLCγ2, when associated with the plasma membrane by virtue of BLNK, gains susceptibility to tyrosine phosphorylation and/or access to its substrate phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2), which in turn generates IP_3 and DAG. This hypothesis predicts that the calcium defect in BLNK-deficient cells should be suppressible by membrane expression of PLCγ2. To test this prediction, a chimera possessing the catalytic domains of PLCγ2 in place of the cytoplasmic domain of FcγRIII (mPLCγ2 shown in Figure 4A) was expressed in BLNK-deficient DT40 cells. Stimulation of the chimeric receptor alone did not induce calcium mobilization (data not shown). Although BCR ligation alone induced a small $[Ca^{2+}]$i increase, coligation of the BCR to the FcγRIII/PLCγ2 chimera resulted in a substantial $[Ca^{2+}]$i increase (Figure 4B). This $[Ca^{2+}]$i increase was more susutained than that upon BCR engagement in wild-type DT40 cells (Figure 3A), suggesting that dissociation of native PLCγ2 from the membrane fraction is likely required for the proper termination of a $[Ca^{2+}]$i increase initiated by BCR ligation.

As shown in Figure 4C, the anti-PLCγ2 Ab recognized two species of FcγRIII/PLCγ2, presumably reflecting posttranslational modifications of this molecule. As expected, coligation of the chimeric receptor and BCR induced tyrosine phosphorylation of FcγRIII/PLCγ2, the upper one of which was more prominent. Collectively, these results indicate that membrane expression of PLCγ2 is sufficient to overcome the inhibition of PLCγ2 activation observed in BLNK-deficient cells.

Ras Activation Still Occurs in the Absence of BLNK

Because the BCR-induced ERK activation is also a downstream event of Syk in DT40 B cells (Jiang et al., 1998), we examined the effect of BLNK on ERK2 activation. BLNK-deficient cells exhibited barely detectable ERK2 activation at 1 min after BCR stimulation, while this activation was observed at 3 min despite less effectively than wild-type cells. In contrast to the inhibition of the ERK2 response in the absence of BLNK, Syk-deficient DT40 cells showed the complete abrogation of the ERK2 response, as reported previously (Jiang et al., 1998) (Figure 5A).

As both PLCγ2 and Ras pathways contribute to ERK2 activation in DT40 cells (Hashimoto et al., 1998), we investigated the mechanism by which BLNK regulates ERK2. To directly determine whether BLNK affects Ras activation, we employed a binding assay developed by Taylor and Shalloway (1996). This assay is based on the observation that Raf protein has high affinity for active Ras-GTP but does not bind the inactive GDP-bound

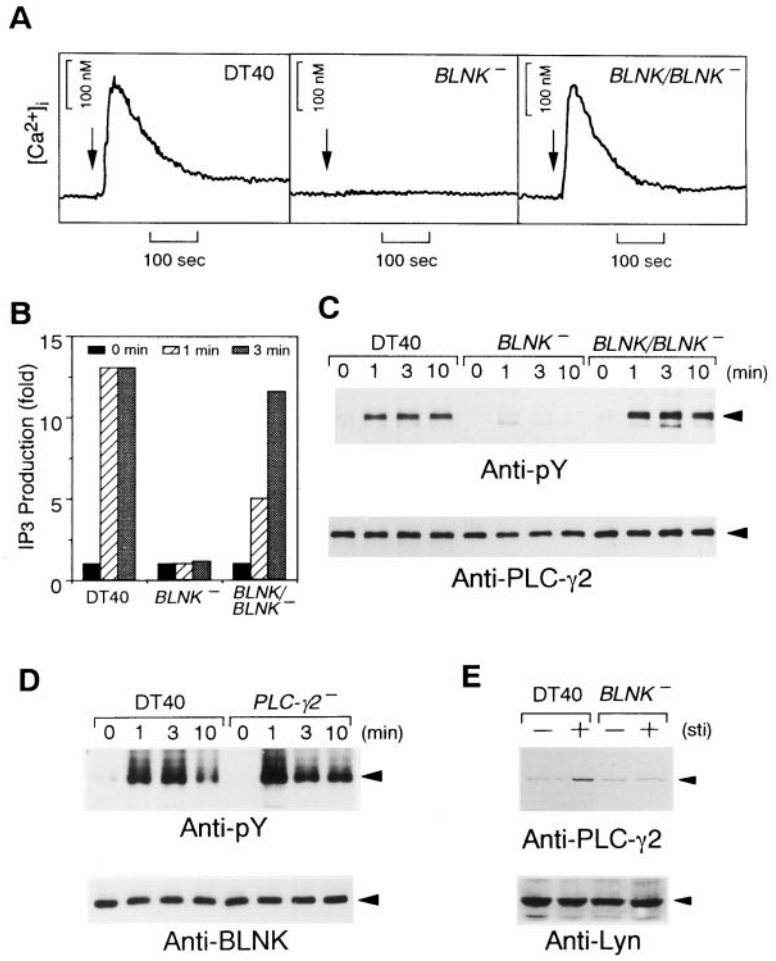

Figure 3. Characterization of BLNK-Deficient DT40 Cells

(A) Calcium mobilization analysis. Intracellular free calcium levels in Fura-2-loaded cells were monitored by a spectrophotometer after stimulation with M4 (2 μg/ml). Arrows indicate the time point for adding M4 Ab.

(B) BCR-induced IP_3 generation. Cells (2×10^6) were stimulated with MAb M4 (10 μg) for indicated time, and IP_3 productions were measured. Data are shown as fold increase of the value before stimulation with M4.

(C) Tyrosine phosphorylation of PLCγ2. At the indicated time points after M4 stimulation (4 μg/ml), immunoprecipitates with anti-PLCγ2 Ab (1×10^7 cells/lane) were separated on 7% SDS-PAGE gel and analyzed by Western blotting with 4G10 (upper). The same filter was reprobed with anti-PLCγ2 Ab (bottom).

(D) Analysis of BLNK phosphorylation in wild-type and PLCγ2-deficient DT40 cell. Cells (5×10^6 cells/lane) were similarly analyzed as in (C) using anti-BLNK Ab and 8% SDS-PAGE gel.

(E) Subcellular analysis of PLCγ2. Wild-type and BLNK-deficient DT40 cells were stimulated by M4 for 3 min (+) or left unstimulated (-). Membrane fractions were analyzed by Western blotting with anti-PLCγ2 Ab (upper) and anti-Lyn Ab (bottom).

form of Ras. BCR crosslinking of wild-type and BLNK-deficient cells both resulted in increased Ras-GTP, though the fold increase observed in the BLNK-deficient cells was somewhat lower than that in wild-type cells (Figure 5B). Hence, the significant attenuation in ERK2 activation in BLNK-deficient cells could not be accounted for by the defect of Ras activation in the mutant cells.

In order to test whether the defect in ERK2 activation by loss of BLNK was due to a defect in the PLCγ2-mediated signaling, we examined whether or not coligation of BCR and FcγRIII/PLCγ2 in BLNK-deficient cells overcomes the ERK2 inhibition. BCR-ligation alone showed a similar degree of inhibition in ERK2 activation (data not shown), while BCR-mediated ERK2 activation was restored in the mutant cells after coligation of these receptors (Figure 5A), indicating that PLCγ2 activation is sufficient to restore the ERK2 response in the absence of BLNK. Together, these data demonstrate that the attenuation in ERK2 activity observed in the BLNK-deficient cells is likely due to a defect in PLCγ2-dependent contribution to ERK2 activation. While the effect on ERK2 activation by BLNK was primarily mediated through PLCγ2, Ras undoubtedly also contributes to full ERK2 activity, as expression of a dominant-negative form of Ras (RasN17) completely abolished the residual ERK2 activity observed in BLNK-deficient cells (Figures 5A and 5C).

BLNK Is Required for the Rac1-JNK Pathway

JNK and p38 responses require Rac1, in addition to PLCγ2 activation. In fact, either expression of a dominant-negative form of Rac1 or loss of PLCγ2 almost completely abolishes the BCR-mediated activation of JNK and p38 (Hashimoto et al., 1998). Thus, to examine the role of BLNK in a Rac1-dependent pathway, we analyzed JNK and p38 responses in wild-type and BLNK-deficient DT40 cells. As shown in Figure 6, BCR-mediated activation of both JNK and p38 was completely abrogated in BLNK-deficient DT40 cells. Using a similar approach for exploring the contribution of PLCγ2 to ERK2 activation, we determined whether coligation of BCR and FcγRIII/PLCγ2 can restore the JNK and p38 responses in BLNK-deficient cells. In contrast to restoration of the ERK2 response by coligation of BCR and FcγRIII/PLCγ2 (Figure 5A), coligation of these receptors was unable to restore the BCR-mediated activation of both JNK and p38. Thus, we conclude that loss of BLNK affects not only the PLCγ2 pathway but also the Rac1-dependent pathway.

Discussion

A striking feature of BCR signaling is that a variety of distinct signaling pathways become activated by multiple cytoplasmic PTKs (DeFranco, 1997). Thus, one of

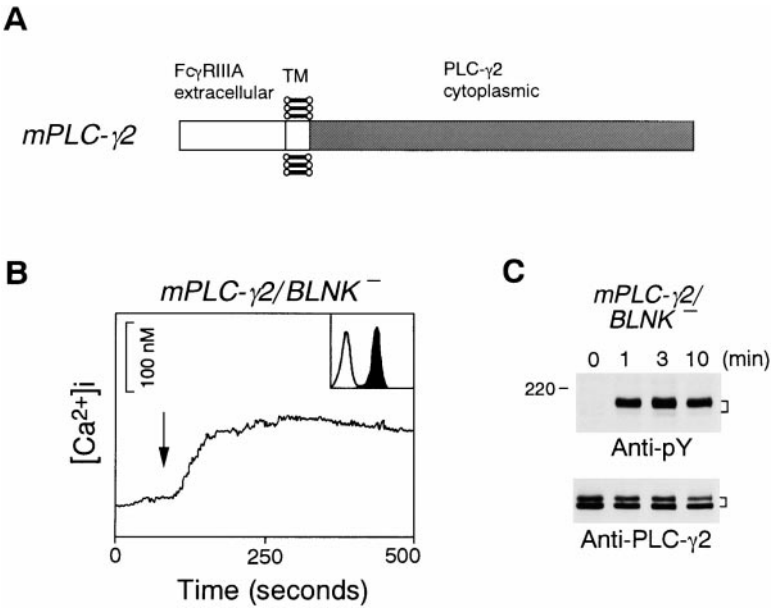

Figure 4. Expression of Membrane-Localized PLCγ2 Suppresses BLNK Inhibition

(A) Schematic diagram of membrane PLCγ2 chimera (mPLCγ2). This is composed of the extracellular domain of human FcγRIIIA (amino acids 1–212) (Ravetch and Perussia, 1989), the transmembrane domain of the human T cell receptor ζ chain (amino acids 30–58) (Weissman et al., 1988), and the complete rat PLCγ2 (Emori et al., 1989) as a cytoplasmic domain.

(B) Calcium mobilization after coligation of BCR and membrane PLCγ2 chimera. For coligation, rabbit anti-mouse IgM was added prior to stimulation with M4. Arrow indicates the addition of M4. Surface expression level of FcγRIII is indicated in an inset box.

(C) BCR-induced tyrosine phosphorylation of mPLCγ2 in BLNK-deficient DT40 cells. After coligation, immunoprecipitates with anti-PLCγ2 Ab were analyzed by Western blotting with 4G10 (upper) or anti-PLCγ2 Ab (bottom).

the key questions is the mechanism by which the BCR-activated PTKs activate the appropriate subset of signaling pathways within the cell. In the case of Syk, at least three signaling pathways (PLCγ2, Ras, and Rac1-JNK) have been identified as its downstream effectors (Takata et al., 1994; Hashimoto et al., 1998; Jiang et al., 1998). In this study, we provide genetic evidence that BLNK functions as a coupling molecule to PLCγ2 and Rac1 pathways, but not to the Ras pathway.

A model for the mechanism of BLNK-mediated activation of PLCγ2, based on the data presented here, is shown in Figure 7. Previous studies have shown that Syk is essential for tyrosine phosphorylation of both BLNK and PLCγ2 upon BCR engagement, since Syk-deficient DT40 cells fail to phosphorylate these molecules (Takata et al., 1994; Fu et al., 1998). In addition, the data shown in Figures 3C and 3D indicate that tyrosine phosphorylation of BLNK by Syk is an upstream event to PLCγ2 phosphorylation. Thus, these findings suggest that the phosphorylated BLNK brings PLCγ2 into close proximity with the activated Syk and thereby facilitates the tyrosine phosphorylation and subsequent activation of PLCγ2. Consistent with this model, a membrane chimera FcγRIII/PLCγ2 was able to overcome the defects of PLCγ2 phosphorylation and its subsequent activation observed in BLNK-deficient cells, when cross-linked to the BCR (Figure 4). The observation that the BCR-mediated translocation of PLCγ2 to the membrane fraction is inhibited by loss of BLNK (Figure 3E), together with the evidence of colocalization of BLNK with Syk (C. F. and A. C. C., unpublished data), further supports this model.

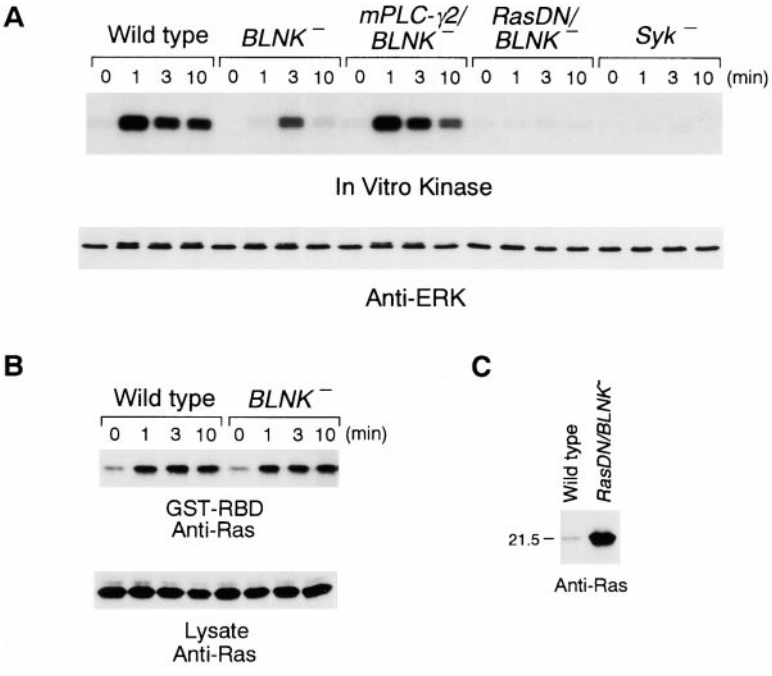

Figure 5. ERK2 Responses upon BCR Engagement

(A) Various DT40 cells were stimulated with M4 (4 μg/ml) for indicated time. For mPLCγ2/BLNK⁻, cells were incubated with anti-mouse IgM (10 μg/ml) for 5 min before M4 simulation. ERK2 was immunoprecipitated and the precipitates were assayed for kinase activity using GST-Elk1 fusion protein as a substrate. The kinase reaction products were resolved by 12.5% SDS-PAGE gel and autoradiogramed. The protein levels in immunoprecipitates by Western blot analysis were shown in a lower panel.

(B) Analysis of Ras activation by the Ras-GTP method. M4 stimulated cell lysates were subjected to affinity precipitation with GST-RBD. Activated Ras proteins were detected by Western blotting with anti-Ras Ab (upper). Similarly, the total amount of Ras protein was determined using the cell lysate (bottom).

(C) Ras protein expression in BLNK-deficient cells expressing RasN17. Expression was measured by Western blot analysis using anti-Ras Ab.

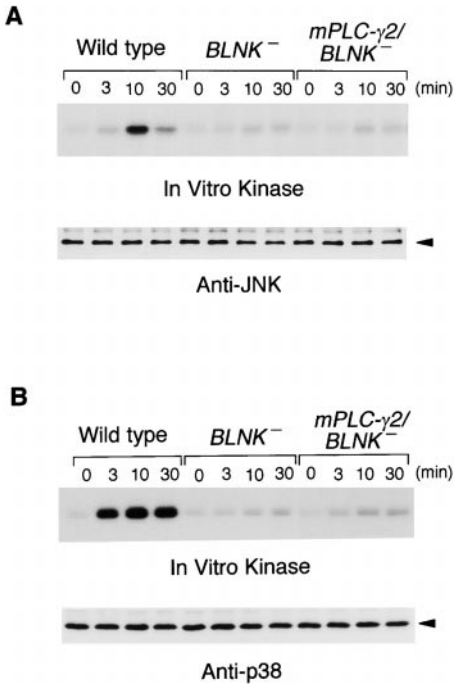

Figure 6. BCR-Induced JNK and p38 Activation in BLNK-Deficient DT40 Cells

Stimulation was carried out as in Figure 5A. After M4 (4 μg/ml) stimulation cell lysates were immunoprecipitated with anti-JNK1 (A) and anti-p38 Ab (B), kinase activities were assayed as in Figure 5A using GST-c-Jun and GST-ATF2 as substrates, respectively.

Btk, in addition to Syk, has been shown to be required for maximum tyrosine phosphophorylation of PLCγ2 upon antigen receptor engagement and its full activation (Takata and Kurosaki, 1996; Fluckiger et al., 1998). Since BCR-induced tyrosine phosphorylation of BLNK normally occurs in Btk-deficient DT40 cells (Fu et al., 1998), we propose that after PLCγ2 is recruited to the membrane fraction by BLNK, the membrane-associated Btk is then able to phosphorylate tyrosine residues on PLCγ2, leading to its full activation. Since Btk SH2 domain, in addition to its PH domain, is required for full PLCγ2 phosphorylation in B cells (Takata and Kurosaki, 1996), Btk SH2 domain might be recruited to tyrosine-phosphorylated BLNK, allowing Btk to phosphorylate PLCγ2.

As BLNK is structurally related to SLP-76, BLNK likely plays a functionally similar role in B cells as SLP-76 plays in T cells. The calcium defect observed in the SLP-76-deficient Jurkat T cell line (Yablonski et al., 1998), however, is not so complete as the phenotype observed in the BLNK-deficient DT40 B cells. This difference may be due to the residual expression of SLP-76 in the mutant Jurkat cell line, as mentioned in their report. Another possibility is that LAT, which is expressed in T but not in B cells (Weber et al., 1998; Zhang et al., 1998), cooperates with SLP-76 in regulating the calcium signaling pathway. Thus, in the case of T cells, the existence of LAT may rescue the calcium defect to some extent even in the absence of SLP-76. Additional studies are underway to further define the functional parallels between the use of these adaptor proteins in T and B cells.

We have previously shown that the synergistic action of Ras and PLCγ2 pathways is required for BCR-induced ERK2 response in DT40 cells. The ERK2 response is partially blocked by loss of PLCγ2 or expression of RasN17, while this response is completely abrogated by introduction of RasN17 into the PLCγ2-deficient mutant (Hashimoto et al., 1998). Thus, the partial inhibition of BCR-mediated ERK2 response in BLNK-deficient DT40 cells can be accounted for by the defect of PLCγ2 pathway in this mutant cell. This explanation is supported by (1) Ras activation even in the absence of BLNK, assessed by binding to GST-Raf; (2) complete inhibition of the ERK2 response by the introduction of RasN17 into the mutant cells; and (3) restoration of the ERK2

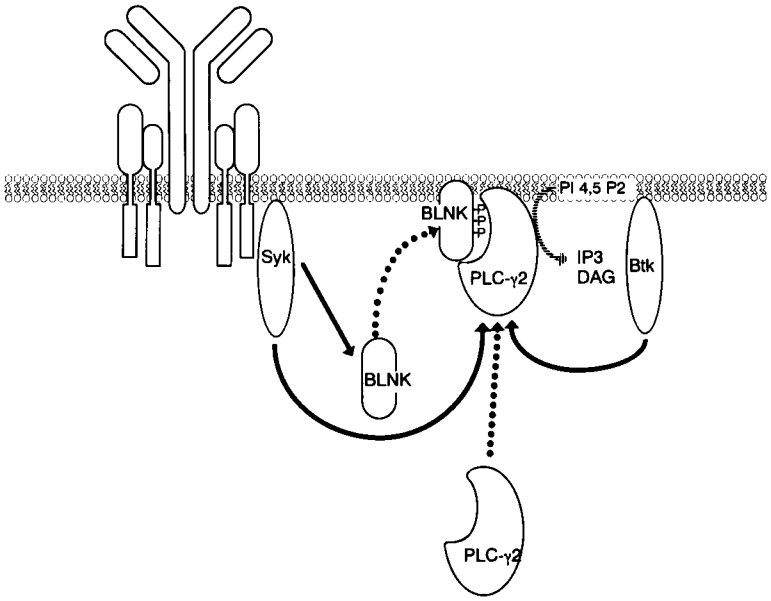

Figure 7. Model for the Mechanism of BLNK-Mediated PLCγ2 Activation upon BCR Stimulation

Upon BCR engagement, activated Syk phosphorylates BLNK, leading to its translocation to the membrane. Phosphorylated BLNK brings PLCγ2 into the close proximity with the activated Syk in the membrane, thereby facilitating tyrosine phosphorylation of PLCγ2 by Syk. Membrane-localized Btk, presumably due to interaction between its PH domain and PI 3,4,5-P_3 (Salim et al., 1996; Rameh et al., 1997; Bolland et al., 1998; Fluckiger et al., 1998), further phosphorylates other tyrosine residues on PLCγ2, resulting in its full activation. Activated PLCγ2 hydrolyzes PI 4,5-P_2 leading to the generation of second messengers, IP_3 and DAG.

response by coligation of BCR and FcγRIII/PLCγ2 chimera in the mutant cells. Given the evidence that translocation of Grb2 from the cytoplasmic to the membrane fraction is critical for Ras activation in B cells (Hashimoto et al., 1998), our data suggest that BLNK is not necessarily required for recruitment of Grb2 to the membrane fraction. Instead, Grb2 might be recruited to another linking molecule, tyrosine phosphorylation of which is mediated presumably by Syk, since BCR-induced ERK2 response is completely abrogated in Syk-deficient DT40 cells (Jiang et al., 1998) (Figure 5).

BLNK-deficient cells were unable to activate JNK or p38 following BCR ligation. Moreover, coligation of the BCR and the FcγRIII/PLCγ2, while sufficient to restore calcium and ERK responses, failed to restore JNK and p38 activation (Figure 6). Together, these results strongly suggest that Rac1 activation is defective in BLNK-deficient DT40 cells. Indeed, expression of a dominant-negative form of Rac1 in DT40 cells abrogates the JNK and p38 responses (Hashimoto et al., 1998). Previous studies have shown that Vav and SLP-76 cooperate in augmenting TCR-mediated IL-2 transcription (Wu et al., 1996; Raab et al., 1997) and that BLNK associates with Vav in activated B cells (Fu and Chan, 1997; Fu et al., 1998; Wienands et al., 1998). Thus, it is most likely that BLNK is required for Vav activity in B cells. Because tyrosine-phosphorylated Vav is able to catalyze GDP/GTP exchange activity on Rac1 (Crespo et al., 1997), one potential mechanism is that BLNK, like its role in PLCγ2 activation, brings Vav to the proximity of activated Syk, thereby allowing Vav to become phosphorylated.

The present findings demonstrate that BLNK functions as a switchboard, allowing PLCγ2 and Rac1-JNK pathways to be turned on. Further selection for PLCγ2 or Rac1 might be determined by phosphorylation of distinct tyrosine residues within BLNK, because BLNK has multiple tyrosine phosphorylation sites. Given that the fate of B cells depends on which set of signaling pathways is connected from the BCR under a given circumstance (Healy and Goodnow, 1998), it is reasonable to anticipate that selection of signaling pathways by BLNK is one of the mechanisms for qualitative regulation of BCR signaling, leading to appropriate biological responses.

Experimental Procedures

Cells and Antibodies

Wild-type and its derivative mutant DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% chicken serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. Anti-BLNK Ab or anti-PLCγ2 Ab was obtained by immunizing rabbits with bacterially expressed GST fusion protein containing chicken BLNK (79–201 amino acid region), or chicken PLCγ2 that is corresponding to rat PLCγ2 818–919 amino acid region (Emori et al., 1989), respectively. The anti-chicken IgM Ab, M4 (μ, κ) (Chen et al., 1982), which was used for stimulation of BCR, anti-phosphotyrosine Ab (4G10), anti-chicken Lyn Ab, rabbit anti-mouse IgM Ab, anti-ERK2 Ab, anti-p38 Ab, and anti-JNK1 Ab were described previously (Takata et al., 1994; Ono et al., 1997; Hashimoto et al., 1998). Anti-Ras Ab and FITC-conjugated anti-human FcγRIII Ab were purchased from Transduction Laboratories and from Pharmingen, respectively.

Expression Constructs and Transfection

Human H-Ras and chicken BLNK (longer form) cDNAs were cloned into expression vector pApuro (Takata et al., 1994). The membrane PLCγ2 chimera cDNA (mPLCγ2 in Figure 4A) was constructed by using PCR method and cloned into pApuro. The cDNA of RasN17 in pApuro vector was already described (Hashimoto et al., 1998). These cDNAs were transfected by electroporation at 550 V, 25 μF, and selected in the presence of 0.5 μg/ml puromycin (Sigma). Expression of transfected cDNA was confirmed by Western blot analysis or FACS analysis (mPLCγ2). Cell surface expression of BCR and FcγRIII were analyzed by FACScan (Becton Dickinson) using FITC-conjugated anti-chicken IgM or FITC-conjugated anti-human FcγRIII Ab, respectively. The x and y axes for the histograms indicate fluorescence intensity (4-decade-log scales) and relative cell number, respectively.

Purification of Chicken pp80

DT40 cells (1.5×10^{10}) were stimulated for 3 min at 2×10^7 cells/ml in RPMI 1640 with M4 (3 μg/ml) at 37°C. Cells were solubilized in 20 ml NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris [pH 7.5], and 1 mM EDTA) containing 50 mM NaF, 10 μM sodium molybdate, 0.1 mM sodium vanadate, and 0.1 mM pervanadate supplemented with protease inhibitors as described previously (Takata et al., 1994). After centrifugation at 13,000 × g, supernatant was precleaned with 5 ml Protein G-Sepharose (Pharmacia) and then loaded onto a 1 ml of 4G10-Protein G-Sepharose column, which was washed sequentially with lysis buffer and PBS buffer both containing 0.1 mM sodium vanadate, 0.1 mM pervanadate, and protease inhibitors. Phosphotyrosine-containing proteins were eluted with PBS containing 50 mM phenylphosphate, 0.1 mM sodium vanadate, 0.1 mM pervanadate, and protease inhibitors. Eluent was passed over Sephadex G-25 column (Pharmacia) for desalting and eluted with NP-40 buffer (0.01% NP-40, 10 mM Tris [pH 7.5], and 1 mM EDTA). Proteins were concentrated, subjected to SDS-PAGE gel, transferred to PVDF membrane (Applied Biosystems), and stained with Ponceau S. The band for pp80 was excised and digested with *Achromobacter* protease I and endoproteinase Asp-N. Digested peptides were chromatographed by reverse-phase HPLC (Wako Pure Chemical), and amino acid sequencing was performed with a gas-phase sequencer (Shimadzu, Model PPSQ-23). We obtained seven peptide sequences as shown in Figure 1A legends.

Generation of BLNK-Deficient DT40 Cells

A 384 bp BLNK cDNA was cloned by RT-PCR method using RNA from DT40 B cells. This fragment was then used as a probe for screening λ ZAP DT40 cDNA library. From 1×10^5 plaques screened, twenty positive clones were identified and seven clones were further characterized. The complete sequence of the longest insert (2417 bp encoding protein of 552 amino acids that contains all seven peptide sequences) has been deposited into GenBank (Accession No. AF089727). Four genomic clones were obtained by screening λ FIXII chicken genomic library (1.3×10^6 plaques) using the same 384 bp DNA fragment as a probe. The targeting vectors, pBLNK-neo and pBLNK-hisD, were constructed by replacing the genomic fragment containing exons that correspond to chicken BLNK amino acid residues 57–213, with neo and hisD cassettes (Takata et al., 1994). These cassettes were flanked by 4.4 kb and 2.2 kb of BLNK genomic sequence on the 5′ and 3′ side, respectively. Transfectants were selected in the presence of G418 (2 mg/ml) and clones were screened by Southern blot analysis. The pBLNK-hisD was again transfected into the neo targeted clone and selected with both G418 (2 mg/ml) and histidiol (1 mg/ml). Introduction of a single copy of each targeting vector was verified by reprobing the blots with internal neo or hisD probe. DT40 cells deficient in PLCγ2 or Syk were described previously (Takata et al., 1994, 1995).

Northern Blot Analysis

RNA was prepared from wild-type and BLNK-deficient DT40 cells using the guanidium thiocyanate method. Total RNA (20 μg) was separated in 1.2% formaldehyde gel, transferred to Hybond-N^+ membrane (Amersham), and probed with ^{32}P-labeled chicken cDNAs BLNK and β-actin (Kost et al., 1983).

Calcium Analysis

Cells (5×10^6) were suspended in PBS containing 20 mM HEPES (pH 7.2), 5 mM glucose, 0.025% BSA, and 1 mM $CaCl_2$, and loaded

with 3 μM Fura-2/AM at 37°C for 45 min. Cells were washed twice and adjusted to 10^6 cells/ml. For mPLCγ2 chimera (Figure 4B), coligation of BCR and the chimera was carried out by adding rabbit anti-mouse IgM (10 μg/ml) followed by anti-chicken IgM MAb, M4 (2 μg/ml). Continuous monitoring of fluorescence from the cell suspension was performed using Hitachi F-2000 fluorescence spectrophotometer (Hitachi) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calibration and calculation of calcium levels were done as described (Grynkiewicz et al., 1985).

IP_3 Generation Assay

Cells (2×10^6) were stimulated with MAb M4 (10 μg) at 37°C for indicated time. Kinetic analysis of IP_3 production was performed using BIOTRAK IP_3 assay system (Amersham) following the manufacturer's protocol.

Immunoprecipitation and Western Blot Analysis

For immunoprecipitation, cells were solubilized in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors (Takata et al., 1994), and precleared lysates were sequentially incubated with proper Abs and protein A-agarose. Lysates or immunoprecipitates were separated by SDS-PAGE gels, transferred to nitrocellulose membranes, and detected by appropriate Abs and ECL system (Amersham).

In Vitro Kinase Assay

The assay conditions were described previously (Hashimoto et al., 1998). In brief, lysates from $2–5 \times 10^6$ cells were immunoprecipitated by 1 μg anti-ERK2 Ab, 1 μg anti-JNK1 Ab, or 1 μg anti-p38 Ab with 40 μl protein-G Sepharose (Pharmacia). Half of immune complexes were used for in vitro kinase assay and the rest for Western blotting. Immunoprecipitates were suspended in 30 μl kinase assay buffer containing [γ-^{32}P]ATP and 5 μM cold ATP. GST-Elk, GST-c-Jun, or GST-ATF2 fusion protein (5 μg each) was added as a substrate for ERK2, JNK1, or p38, respectively. After 20 min incubation at 30°C, the reaction was terminated by the addition of SDS sample buffer followed by boiling for 5 min. The samples were separated by SDS-PAGE gels, dried, and subjected to autoradiography.

Ras-GTP Assay

Bacterially expressed GST-RBD (Ras binding domain: amino acids 1–149 of human cRaf-1 fused to GST) prebounded glutathione-Sepharose beads (15 μl packed beads, 20 μg of protein) were prepared as described (Taylor and Shalloway, 1996). Human H-Ras cDNA was transfected into wild-type and BLNK-deficient DT40 cells. Wild-type and mutant cells expressing similar levels of Ras were selected and used for this assay. M4-stimulated cell lysates in Mg^{2+}-containing lysis buffer (Taylor and Shalloway, 1996) were incubated with the beads for 30 min at 4°C. Bound proteins were eluted with SDS-PAGE sample buffer and resolved on 12.5% SDS-PAGE gel and subjected to Western blotting with anti-Ras Ab.

Subcellular Analysis of PLCγ2

Subcellular fraction was performed at 4°C as described by Bolland et al. (1998) with some modifications. Wild-type or BLNK-deficient DT40 cells (1×10^8 each) were stimulated with M4 (10 μg/ml) for 3 min at 37°C or left unstimulated. Cells were resuspended in 1 ml of hypotonic lysis buffer (10 mM Tris [pH 8.0], 2 mM $MgCl_2$, 1 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium molybdate, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstain, 2 mM benzamidine, 50 mM NaF, and 40 ng/ml PMSF) and Dounce homogenized. Cell lysates were centrifuged at $1,600 \times$ g for 10 min. The resulting supernatants were centrifuged at $100,000 \times$ g for 30 min. Precipitates were washed twice in lysis buffer and resuspended in lysis buffer containing 1% Triton X-100. These resuspensions were centrifuged at $12,000 \times$ g for 20 min. Proteins were separated by 7% SDS-PAGE gel and analyzed by Western blotting using anti-PLCγ2 Ab.

Acknowledgments

We would like to thank R. Goitsuka, T. Nakayama, T. Urano, T. Adachi, and D. Cantrell for providing us with λ ZAP DT40 cDNA library, λ FIXII chicken genomic library, human H-Ras cDNA, chicken SLP-76 cDNA, and GST-RBD expression plasmid, respectively, and A. Kosugi and M. Gold for advice on the subcellular analysis and the Ras-GTP assay. This work was supported by grants to M. I. and T. K. from the Ministry of Education, Science, Sports, and Culture of Japan, to T. K. from Takeda Science Foundation, and to A. C. C. from the National Institutes of Health (R01AI42787 and R01CA71516). A. C. C. is also a Pew Scholar in the Biomedical Sciences.

Received October 5, 1998; revised December 10, 1998.

References

Bolland, S., Pearse, R.N., Kurosaki, T., and Ravetch, J.V. (1998). SHIP modulates immune receptor responses by regulating membrane association of Btk. Immunity *8*, 509–516.

Chen, C.-L.H., Lehmeyer, J.E., and Cooper, M.D. (1982). Evidence for an IgD homologue on chicken lymphocytes. J. Immunol. *129*, 2580–2585.

Coggeshall, K.M., McHugh, J.C., and Altman, A. (1992). Predominant expression and activation-induced tyrosine phosphorylation of phospholipase C-γ 2 in B lymphocytes. Proc. Natl. Acad. Sci. USA *89*, 5660–5664.

Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S., and Bustelo, X.R. (1997). Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature *385*, 169–172.

DeFranco, A.L. (1997). The complexity of signaling pathways activated by the BCR. Curr. Opin. Immunol. *9*, 296–308.

Emori, Y., Homma, Y., Sorimachi, H., Kawasaki, H., Nakanishi, O., Suzuki, K., and Takenawa, T. (1989). A second type of rat phosphoinositide-specific phospholipase C containing a *src*-related sequence not essential for phosphoinositide-hydrolyzing activity. J. Biol. Chem. *264*, 21885–21890.

Fluckiger, A.-C., Li, Z., Kato, R.M., Wahl, M.I., Ochs, H.D., Longnecker, R., Kinet, J.-P., Witte, O.N., Scharenberg, A.M., and Rawlings, D.J. (1998). Btk/Tec kinases regulate sustained increases in intracellular Ca^{2+} following B-cell receptor activation. EMBO J. *17*, 1973–1985.

Fu, C., and Chan, A.C. (1997). Identification of two tyrosine phosphoproteins, pp70 and pp68, which interact with phospholipase Cγ, Grb2 and Vav after B cell antigen receptor activation. J. Biol. Chem. *272*, 27362–27368.

Fu, C., Turck, C.W., Kurosaki, T., and Chan, A.C. (1998). BLNK: a central linker protein in B cell activation. Immunity *9*, 93–103.

Fukazawa, T., Reedquist, K.A., Trub, T., Soltoff, S., Panchamoorthy, G., Druker, B., Cantley, L., Shoelson, S.E., and Band, H. (1995). The SH3 domain-binding T cell tyrosyl phosphoprotein p120. Demonstration of its identity with the c-cbl protooncogene product and in vivo complexes with Fyn, Grb2, and phosphatidylinositol 3-kinase. J. Biol. Chem. *270*, 19141–19150.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. *260*, 3440–3450.

Harmer, S.L., and DeFranco, A.L. (1997). Shc contains two Grb2 binding sites needed for efficient formation of complexes with Sos in B lymphosites. Mol. Cell. Biol. *17*, 4087–4095.

Hashimoto, A., Okada, H., Jiang, A., Kurosaki, M., Greenberg, S., Clark, E.A., and Kurosaki, T. (1998). Involvement of guanosine triphosphatases and phospholipase C-γ2 in extracellular signal-regulated kinase, c-Jun NH_2-terminal kinase, and p38 mitogen-activated protein kinase activation by the B cell antigen receptor. J. Exp. Med. *188*, 1287–1295.

Healy, J.I., and Goodnow, C.C. (1998). Positive versus negative signaling by lymphocyte antigen receptors. Annu. Rev. Immunol. *16*, 645–670.

Hempel, W.M., and DeFranco, A.L. (1991). Expression of phospholipase C isozymes by murine B lymphocytes. J. Immunol. *146*, 3713–3720.

Hutchcroft, J.E., Harrison, M.L., and Geahlen, R.L. (1991). B lymphocyte activation is accompanied by phosphorylation of a 72-kDa protein-tyrosine kinase. J. Biol. Chem. *266*, 14846–14849.

Jackman, J.K., Motto, D.G., Sun, Q., Tanemoto, M., Turck, C.W., Peltz, G.A., Koretzky, G.A., and Findell, P.R. (1995). Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. J. Biol. Chem. *270*, 7029–7032.

Jiang, A., Craxton, A., Kurosaki, T., and Clark, E.A. (1998). Different protein tyrosine kinases are required for B cell antigen receptor-mediated activation of extracellular signal-regulated kinase, c-Jun NH_2-terminal kinase 1, and p38 mitogen-activated protein kinase. J. Exp. Med. *188*, 1297–1306.

Kost, T.A., Theodorakis, N., and Hughes, S.H. (1983). The nucleotide sequence of the chick cytoplasmic β-actin gene. Nucleic Acids Res. *11*, 8287–8301.

Kurosaki, T. (1997). Molecular mechanisms in B cell antigen receptor signaling. Curr. Opin. Immunol. *9*, 309–318.

Kurosaki, T., Johnson, S.A., Pao, L., Sada, K., Yamamura, H., and Cambier, J.C. (1995). Role of the Syk autophosphorylation site and SH2 domains in B cell receptor signaling. J. Exp. Med. *182*, 1815–1823.

Law, C.-L., Chandran, K.A., Sidorenko, S.P., and Clark, E.A. (1996). Phospholipase C-γ1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. Mol. Cell. Biol. *16*, 1305–1315.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D., and Schlessinger, J. (1992). The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell *70*, 431–442.

Nishibe, S., Wahl, M.I., Hernández-Sotomayor, S.M.T., Tonks, N.K., Rhee, S.G., and Carpenter, G. (1990). Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. Science *250*, 1253–1256.

Ono, M., Okada, H., Bolland, S., Yanagi, S., Kurosaki, T., and Ravetch, J.V. (1997). Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. Cell *90*, 293–301.

Panchamoorthy, G., Fukazawa, T., Miyake, S., Soltoff, S., Reedquist, K., Druker, B., Shoelson, S., Cantley, L., and Band, H. (1996). $p120^{cbl}$ is a major substrate of tyrosine phosphorylation upon B cell antigen receptor stimulation and interacts in vivo with Fyn and Syk tyrosine kinases, Grb2 and Shc adaptors, and the p85 subunit of phosphatidylinositol 3-kinase. J. Biol. Chem. *271*, 3187–3194.

Pawson, T., and Schlessinger, J. (1993). SH2 and SH3 domains. Curr. Biol. *3*, 434–442.

Pleiman, C.M., D'Ambrosio, D., and Cambier, J.C. (1994). The B-cell antigen receptor complex: structure and signal transduction. Immunol. Today *15*, 393–399.

Raab, M., da Silvia, A.J., Findell, P.R., and Rudd, C.E. (1997). Regulation of Vav-SLP-76 binding by ZAP-70 and its relevance to TCRζ/CD3 induction of interleukin-2. Immunity *6*, 155–164.

Rameh, L.E., Arvidsson, A.-K., Carraway, K.L., III, Couvillon, A.D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M.P., Ravichandran, K.S., Burakoff, S.J., et al. (1997). A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. J. Biol. Chem. *272*, 22059–22066.

Ravetch, J.V., and Perussia, B. (1989). Alternative membrane forms of FcγRIII (CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. J. Exp. Med. *170*, 481–497.

Reth, M., and Wienands, J. (1997). Initiation and processing of signals from the B cell antigen receptor. Annu. Rev. Immunol. *15*, 453–479.

Richards, J.D., Gold, M.R., Hourihane, S.L., DeFranco, A.L., and Matsuuchi, L. (1996). Reconstitution of B cell antigen receptor-induced signaling events in a nonlymphoid cell line by expressing the Syk protein-tyrosine kinase. J. Biol. Chem. *271*, 6458–6466.

Rowley, R.B., Burkhardt, A.L., Chao, H.-G., Matsueda, G.R., and Bolen, J.B. (1995). Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Ig α/Ig β immunoreceptor tyrosine activation motif binding and autophosphorylation. J. Biol. Chem. *270*, 11590–11594.

Salim, K., Bottomley, M.J., Querfurth, E., Zvelebil, M.J., Gout, I., Scaife, R., Margolis, R.L., Gigg, R., Smith, C.I.E., Driscoll, P.C., et al. (1996). Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. EMBO J. *15*, 6241–6250.

Saxton, T.M., van Oostveen, I., Bowtell, D., Aebersold, R., and Gold, M.R. (1994). B cell antigen receptor cross-linking induces phosphorylation of the $p21^{ras}$ oncoprotein activators SHC and mSOS1 as well as assembly of complexes containing SHC, GRB-2, mSOS1, and a 145-kDa tyrosine-phosphorylated protein. J. Immunol. *153*, 623–636.

Smit, L., de Vries-Smits, A.M.M., Bos, J.L., and Borst, J. (1994). B cell antigen receptor stimulation induces formation of a Shc-Grb2 complex containing multiple tyrosine-phosphorylated proteins. J. Biol. Chem. *269*, 20209–20212.

Takata, M., and Kurosaki, T. (1996). A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-γ2. J. Exp. Med. *184*, 31–40.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kurosaki, T. (1994). Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways. EMBO J. *13*, 1341–1349.

Takata, M., Homma, Y., and Kurosaki, T. (1995). Requirement of phospholipase C-γ2 activation in surface immunoglobulin M-induced B cell apoptosis. J. Exp. Med. *182*, 907–914.

Taylor, S.J., and Shalloway, D. (1996). Cell cycle-dependent activation of Ras. Curr. Biol. *6*, 1621–1627.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight martrix choice. Nucleic Acids Res. *22*, 4673–4680.

Weber, J.R., Ørstavik, S., Torgersen, K.M., Danbolt, N.C., Berg, S.F., Ryan, J.C., Tasken, K., Imboden, J.B., and Vaage, J.T. (1998). Molecular cloning of the cDNA encoding pp36, a tyrosine-phosphorylated adaptor protein selectively expressed by T cells and natural killer cells. J. Exp. Med. *187*, 1157–1161.

Weissman, A.M., Hou, D., Orloff, D.G., Modi, W.S., Seuanez, H., O'Brien, S.J., and Klausner, R.D. (1988). Molecular cloning and chromosomal localization of the human T-cell receptor ζ chain: distinction from the molecular CD3 complex. Proc. Natl. Acad. Sci. USA *85*, 9709–9713.

Wienands, J., Schweikert, J., Wollscheid, B., Jumaa, H., Nielsen, P.J., and Reth, M. (1998). SLP-65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. J. Exp. Med. *188*, 791–795.

Wu, J., Motto, D.G., Koretzky, G.A., and Weiss, A. (1996). Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. Immunity *4*, 593–602.

Yablonski, D., Kuhne, M.R., Kadlecek, T., and Weiss, A. (1998). Uncoupling of nonreceptor tyrosine kinases from PLC-γ1 in an SLP-76-deficient T cell. Science *281*, 413–416.

Yamada, T., Taniguchi, T., Yang, C., Yasue, S., Saito, H., and Yamamura, H. (1993). Association with B-cell-antigen receptor with protein-tyrosine kinase $p72^{syk}$ and activation by engagement of membrane IgM. Eur. J. Biochem. *213*, 455–459.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R.P., and Samelson, L.E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell *92*, 83–92.

GenBank Accession Number

The GenBank accession number for the sequence reported in this paper is AF089727.